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Supplemental Information

CENP-C Is a Structural Platform for Kinetochore Assembly

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A

Protein A alone				
#	CG	Protein	Score	Cover. %
1	9277	β-Tubulin at 56D	152	19
2	1913	α-Tubulin	135	6
3	4147	Heat shock protein cognate 3	129	4
4	12101	Heat shock protein 60	118	10
5	31022	Prolyl-4-hydroxylase αEFB	94	2
6	4169	CG4169	67	3
7	8947	26-29kD-proteinase	57	2
8	18572	Rudimentary	54	1
9	33332	CG33332	51	5
10	18255	Stretchin-Mlck	47	below 1
11	8280	Elongation factor 1α48D	45	5
12	2774	CG2774	45	2
13	10827	CG10827	42	2
14	17246	Succinate dehydrogenase A	42	1
15	5504	Lethal (2) tumorous imaginal discs	41	3
16	15144	CG15144	41	1
17	31915	CG31915	41	2
18	3571	KLHL18	40	2

B

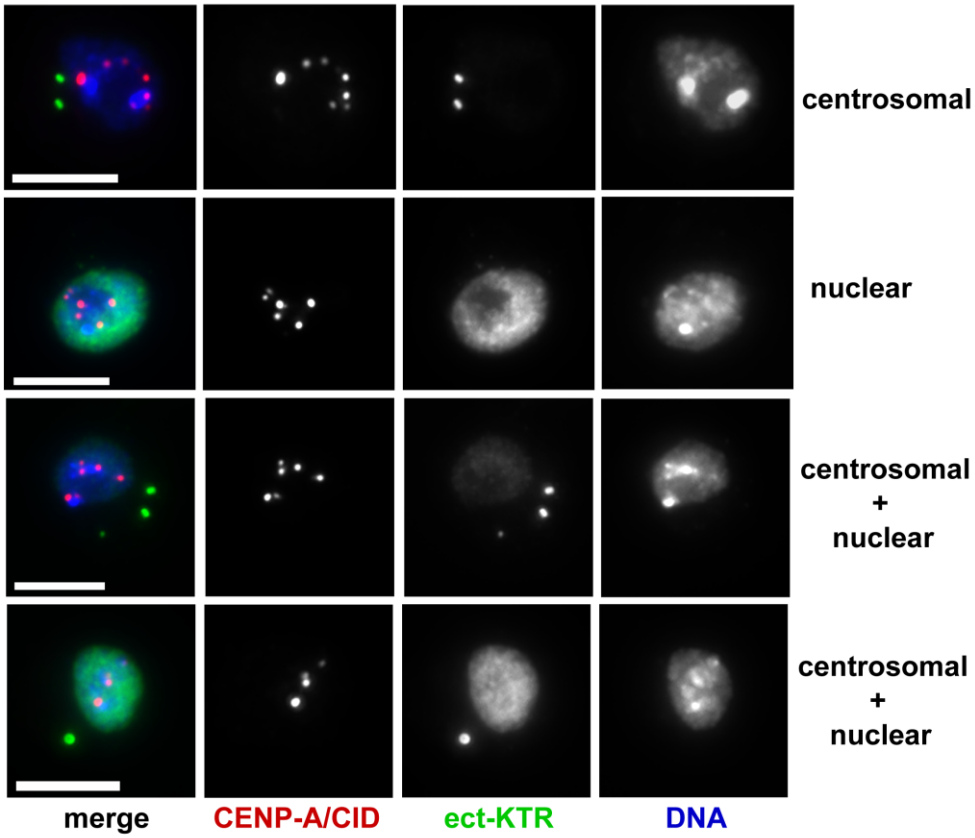

Figure S1.

Figure S1. Control Experiments Related to Figure 1

(A) Proteins identified in the affinity purification of the Protein A alone (not fused with any other protein) expressed in Dmel-2 cells.

(B) Examples of different localization patterns of ect-KTR. Interphase cells from three major categories are shown: centrosomal localization only, nuclear localization only, and both centrosomal and nuclear localization of ect-KTR fusion protein within the same cell. Expression levels varied significantly from cell to cell. Cells were costained with anti-GFP, anti-CENP-A/CID antibodies, and DAPI (DNA). Scale bar represents 5 μm.

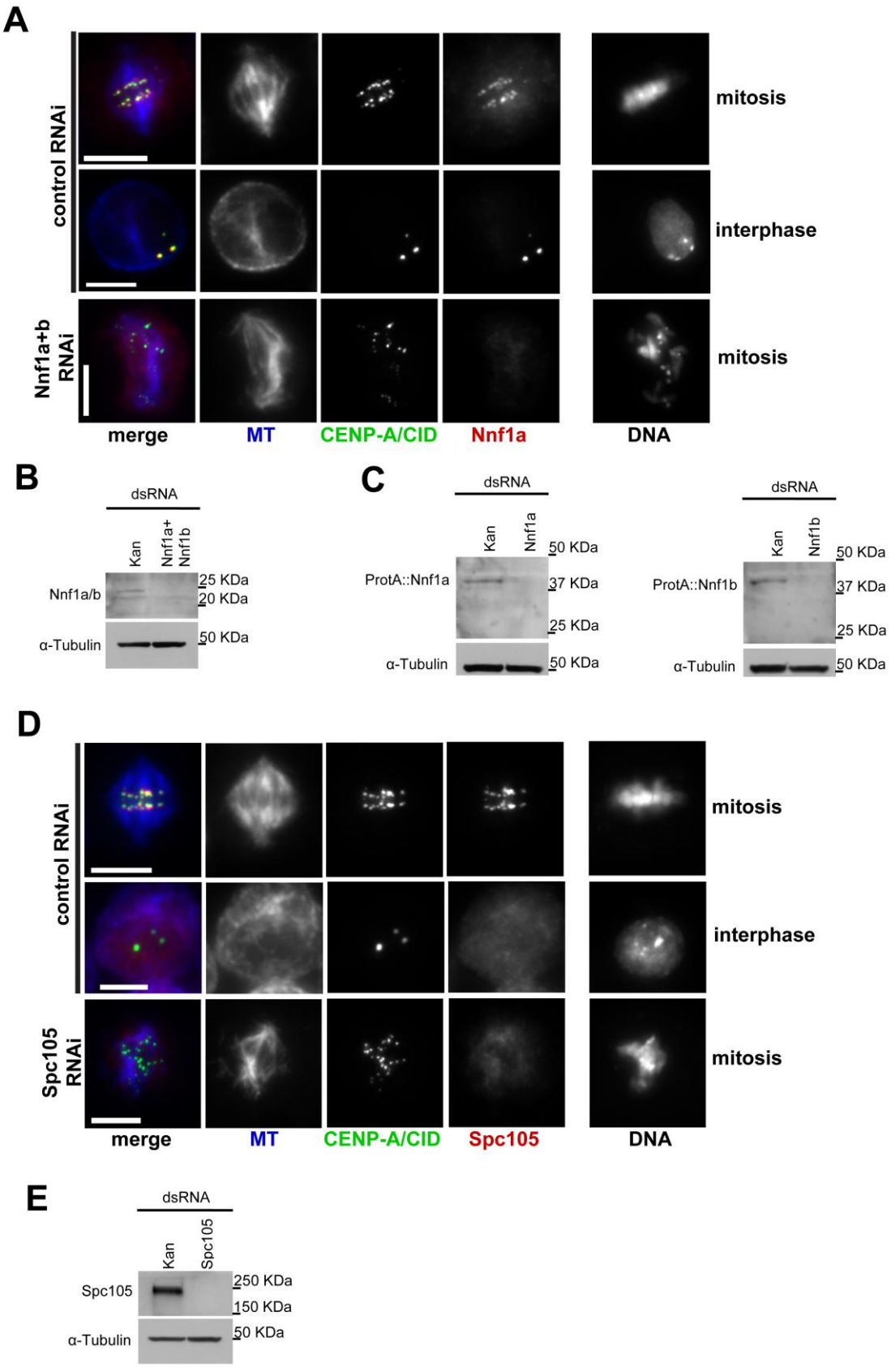
A
control RNAi
Nnf1a+b RNAi
mitosis
interphase
mitosis
merge
MT
CENP-A/CID
Nnf1a
DNA
B
dsRNA
Kan
Nnf1a+ Nnf1b
Nnf1a/b
α-Tubulin
25 KDa
20 KDa
50 KDa
C
dsRNA
Kan
Nnf1a
ProtA::Nnf1a
α-Tubulin
50 KDa
37 KDa
25 KDa
50 KDa
dsRNA
Kan
Nnf1b
ProtA::Nnf1b
α-Tubulin
50 KDa
37 KDa
25 KDa
50 KDa
D
control RNAi
Spc105 RNAi
mitosis
interphase
mitosis
merge
MT
CENP-A/CID
Spc105
DNA
E
dsRNA
Kan
Spc105
Spc105
α-Tubulin
250 KDa
150 KDa
50 KDa

Figure S2.

Figure S2. Characterization of New Antibodies Used in the Experiments Presented in Figure 2

(A) Dmel-2 cells stained with the antibody raised against *Drosophila* Nnf1a. Cells were counterstained with the anti-CENP-A/CID and anti-αTubulin antibodies. RNAi targeting both Nnf1a and Nnf1b eliminates the Nnf1 staining and proves the antibody specificity. Importantly, the anti-Nnf1a antibody staining indicates Nnf1a and/or Nnf1b to be present on centromeres not only in mitosis, but also during interphase (We recently described similar findings for Mis12, but not for Nsl1 [27]). Scale bar represents 5 μm.

(B) Western blots showing endogenous Nnf1a/b proteins detected by this antibody and their depletion by combined *Nnf1a* and *Nnf1b* dsRNA but not by *kanamycin resistance* (kan) dsRNA.

(C) Western blots of lines over-expressing protein A fusions with either Nnf1a (left panel) or Nnf1b (right panel). Both are equally well recognized by the new anti-Nnf1a antibody and equally well depleted by the appropriate dsRNA treatment.

(D) Cells stained with the antibody against *Drosophila* Spc105 protein (costaining as in A). Spc105 is present on kinetochores only during mitosis. Scale bar represents 5 μm.

(E) Western blotting showing the endogenous Spc105 protein and its depletion by RNAi.

For details about production of antibodies and recommended dilutions, see Supplemental Experimental Procedures. Knockdown experiments were performed exactly as described elsewhere [9].

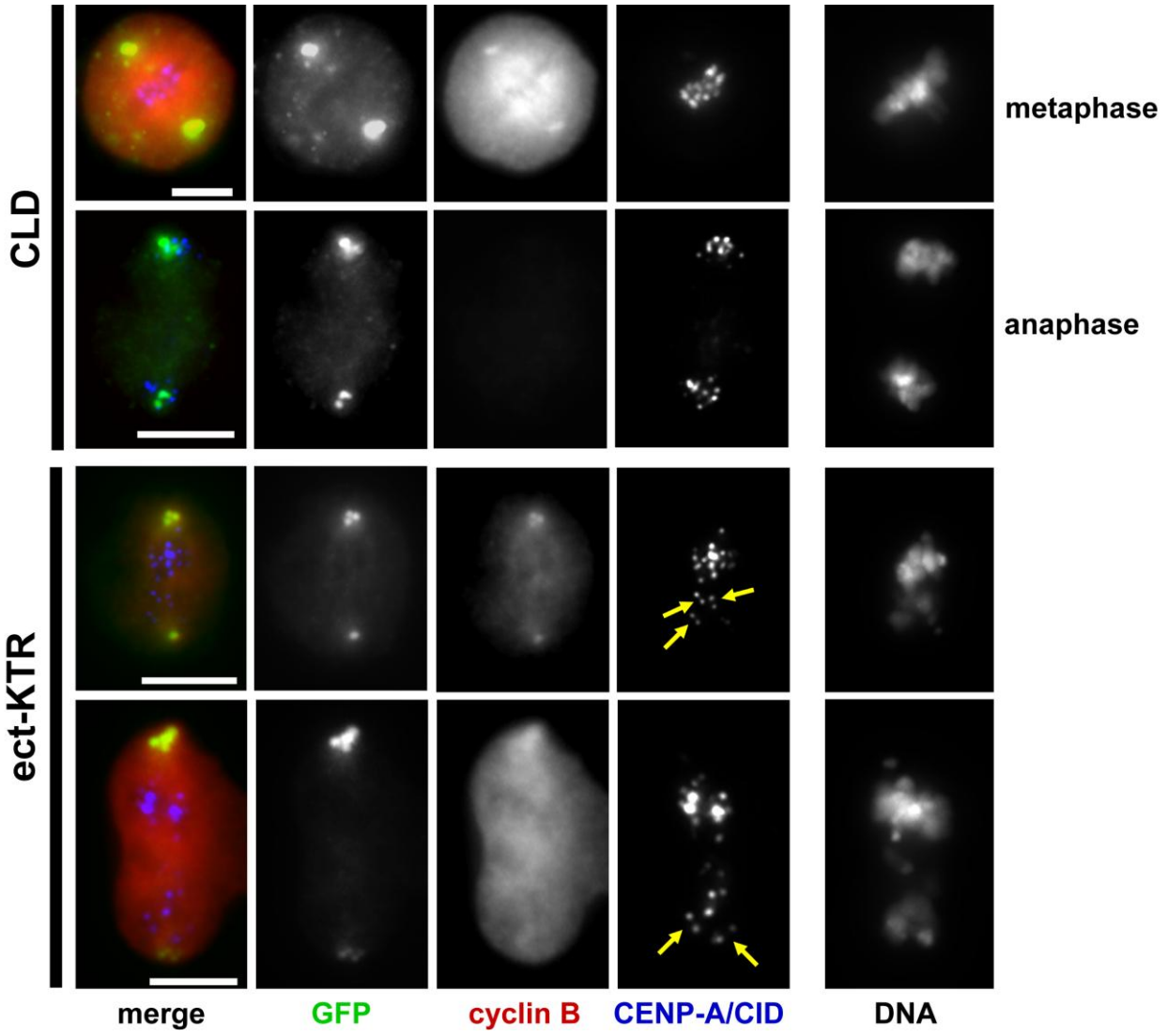

Figure S3. Staining with Anti-Cyclin B Antibody Reveals that ect-KTR Cells with Mitotic Phenotypes Depicted in Figure 4 Are Blocked in a Preanaphase Stage

Staining with anti-GFP antibody ensures that CLD and ect-KTR cells are in fact transgenic. DNA staining (DAPI; not merged) shows scattered chromosome phenotype in ect-KTR cells and a normal distribution of chromosomes in CLD cells. Cyclin B levels in ect-KTR cells are strongly elevated, suggesting that these cells did not enter anaphase. This conclusion is supported by the presence of "double punctae" of anti-CENP-A/CID antibody staining indicative of conjoined chromatids (yellow arrows). Scale bar represents 5 µm.

Table S1. Primers Used for Cloning

Name	Sequence	Comment
CENPC-Not-U1	ATAAGAAGCGGCCGCATGTCGAAGCCCCAGAACAACGAC	Upstream primer for the full length CENP-C
CENPC-Xba-L1	GCTCTAGAACTGCGTATACACATCAGCACACT	Downstream primer for the full length CENP-C
GW-CENPC-5pri-U1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCGAAGCCCCAGAACAACGACA	Upstream primer for the amplification of CENPC_N for cloning in the Gateway system (entry clone)
GW-CENPC-5pri-L1	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAATTCTCTTCTTCAACATATTCG	Downstream primer for the amplification of CENPC_N for cloning in the Gateway system (entry clone)
GW-CENPC-3pri-U1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGCAACAATGAGCAATCAAGGA	Upstream primer for the amplification of CENPC_C for cloning in the Gateway system (entry clone)
GW-CENPC-3pri-L1	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAACTGCGTATACACATCAGCACA	Downstream primer for the amplification of CENPC_C for cloning in the Gateway system (entry clone)
GW-SAK_ABD-NU1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCAGAGCCAAATTGGCCAGGAG	Upstream primer for amplification of CLD (fragment of Plk4/SAK) for the cloning in the Gateway system
GW-SAK_ABD-NL1	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATACATCTGTTATAGGTCTTCGT	Downstream primer for amplification of CLD (fragment of Plk4/SAK) for the cloning in the Gateway system
CENPC5p-U1	ATGTCGAAGCCCCAGAACAAC	Upstream and downstream primers for the first step amplification in the PCR-driven overlap extension method for CENPC_N
CENPC5p-CTDjoin-L1	TCCTGGCCAATTTGGCTCTGCATTCTCTTCTTCAACATATTC	
CENPC5p-CTDjoin-U1	GAATATGTTGAAGAAGAGAATGCAGAGCCAAATTGGCCAGGA	Upstream and downstream primers for the first step amplification in the PCR-driven overlap extension method for CLD
CTD-L1	CTATACATCTGTTATAGGTCTT	
GW-CENPC_5+CTD-NU1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCGAAGCCCCAGAACAACGACA	Upstream and downstream primers for the second step amplification in the PCR-driven overlap extension method: the fusion CENPC_N+CLD gets amplified with ends suitable for cloning in the Gateway system (entry clone)
GW-CENPC_5+CTD-NL1	GGGGACCACTTTGTACAAGAAAGCTGGGTACTATACATCTGTTATAGGTCTTCGT	
GW-5148-NU1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGAATGCGGTGGTGGACGAGG	Upstream primer for the amplification of CAL1 for cloning in the Gateway system (entry clone)
GW-5148-NL1	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTTGTCACCGGAATTATTCTCG	Downstream primer for the amplification of CAL1 for cloning in the Gateway system (entry clone)

Supplemental Experimental Procedures

cDNAs, Constructs, and Vectors

cDNA for CENP-C was amplified from the total cDNA prepared according to the standard procedure described in the Superscript Reverse Transcriptase Kit (Invitrogen). cDNAs coding for Nuf2 and Ndc80 were obtained from the Drosophila Genomics Resource Center (DGRC) and described before [9]. cDNA coding for CAL1 was obtained from Ronald Vale laboratory (HHMI, UCSF) [35]. Primers for the amplification of the full-length sequences and the fragments of CENP-C used for making constructs are given in the Supplemental Materials section. cDNAs coding for chimeric (fusion) proteins were made using two-step PCR via PCR-driven overlap extension method [36]. All the expression vectors used in this study utilize the inducible metallothionein promoter, are in the Gateway system and were described previously [9, 11, 17]. Constructs were sequenced before being used for cell transfections.

Cells and Expressions

Dmel-2 cells (Invitrogen) were grown in Express Five SFM (Invitrogen) media according to standard procedures. Stable cell lines were made exactly according to [9] and [17], with one exception: FuGENE HD (Roche) was used here as a transfection reagent. Overnight-long induction of expression was achieved by adding cupric sulfate to cell culture media to a final concentration of 0.5 mM. For the purpose of imaging cells were transferred onto glass coverslips, fixed with the formaldehyde or ice-cold methanol and stained as described previously [9].

Affinity Purifications and Mass Spectrometry

Detailed protocols for making IgG-beads and for the affinity purification from Dmel-2 cells were published previously [17]. Peptides mixtures were analyzed by LC-MS-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using Nano-Acquity (Waters) LC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA). Prior to the analysis, proteins were subjected to standard ”in-solution digestion” procedure during which proteins were reduced with 100 mM DTT (for 30 minutes at 56°C), alkylated with 0.5 M iodoacetamide (45 minutes in darkroom at room temperature) and digested overnight with trypsin (sequencing Grade Modified Trypsin - Promega V5111). Peptide mixture was applied to RP-18 precolumn (nanoACQUITY Symmetry® C18 – Waters 186003514) using water containing 0,1% TFA as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18 - Waters 186003545) using an acetonitrile gradient (0 % - 60 % AcN in 120 minutes) in the presence of 0.05% formic acid with the flowrate of 150 nl/min. Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis.

Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, on-site license) against FlyBase database. Search parameters for precursor and product ion mass tolerance were 20 ppm and 0.6 Da, respectively, with search parameters set as follows: one missed semiTrypsin cleavage site allowed, fixed modification of cysteine by carbamidomethylation and variable modification of lysine carbamidomethylation and methionine oxidation. Peptides with Mascot score exceeding the threshold value corresponding to <5% false positive rate, calculated by Mascot, were considered to be positively identified.

In Vitro Binding Assay

cDNAs coding for CENPC-N and CENPC-C were recombined into pDEST15 vector (Invitrogen) and expressed using Rosetta 2(DE3)pLysS cells (Novagen). GST fusions were purified using Glutathione Sepharose 4b resin (GE Healthcare) according to the manufacturer's instructions. The expression of Mis12 complex components [9] and the binding assay were performed as described elsewhere [11].

Antibodies and Imaging

The following previously published or commercially available primary antibodies were used in this study: chicken α-CENP-A/CID [9] 1:1000; mouse α-lamin B (T47) [37] 1:20; mouse monoclonal α-GFP (Roche) 1:500; rabbit α-RFP (Abcam) 1:100; chicken α-dPlp [38] 1:1000; rabbit α-Spd2 (our laboratory) 1:100; rabbit α-Mis12 [27] 1:500; rabbit α-Nsl1 [27]; rat α-alpha tubulin (YL1/2; SEROTEC) 1:50; rabbit α-CENP-meta (gift from Don Cleveland's lab, UCSD) 1:50; rabbit α-Mad2 (gift from David Sharp's lab, Einstein College of Medicine) 1:50; rabbit α-BubR1 [27] 1:500; mouse α-phosphoH3 (Ser10) (Cell Signaling) 1:1000. Additionally, new primary antibodies against *Drosophila* Nnf1a and Spc105 were raised in sheep. Recombinant, C-terminal 6xHis tagged 1-585aa region of Spc105 and C-terminal 6xHis tagged full length Nnf11a were expressed in *Escherichia coli*. The insoluble proteins were dissolved in 8M Urea, affinity purified, refolded and used for immunizations. 72 days bleeds of the sheep were found to be the best in control Western blotting and immunohistochemistry experiments. In this study Nnf1a serum was used in dilution 1:2000 for Western blotting and 1:500 for immunohistochemistry. Spc105 serum was used in dilution 1:3000 for Western blotting and 1:1000 for immunohistochemistry.

Secondary antibodies were either from Invitrogen or Jackson ImmunoResearch laboratories and were all used at 1:300 dilution.

Images were taken using Zeiss Axiovert 200M microscope (100x objective) equipped with the Photometrics CoolSNAP HQ2 camera. They were captured and processed using MAG Biosystems Software – Metamorph (Molecular Devices, Inc). We did not observe any bleed-through of fluorescence between channels when single fluorescent probes were imaged.

Signal intensities were measured using ImageJ software as described elsewhere [39].

Supplemental References

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